



CRP in Relation To CVD

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Abstract: Cardiovascular diseases are leading to death in both men and women. One of the risk factors mainly involved in CVD and CRP as inflammatory markers. Atherosclerosis is a disease characterized by chronic inflammation and is now widely accepted to be inflammatory diseases. Clinical manifestations include coronary heart disease [CHD], cerebrovascular disease and peripheral vascular disease.

In this study focus on CRP, this protein will be expressed on CVD patients and the relationship between Insulin resistance leads to cardiovascular disease and diabetic diseases occurs in young age & adult age peoples were living worldwide.

The CVD patient and normal sample were collected respectively in hospital. These samples were estimated by protein estimation and analysis of CRP using SDS PAGE gel electrophoresis and assay method of western blot done by the color development system using primary & secondary antibody and also DAB kit. The measurements of BMI correlate to the CRP and INSULIN level. Increased BMI that can lead to obesity occurs in both men & women. The CRP protein level increases the chance of cardiovascular disease and insulin level also increased, the patient should develop diabetes. These diseases are analyzed by diagnostic methods.

In human CRP was synthesized by hepatocytes and secreted into serum and also whether CRP as a human acute phase protein synthesis by PBMC and isolate the lymphocytes in cultured medium and protein was isolated & estimated due to analysis of CRP using SDS PAGE Gel Electrophoresis. From the preliminary results, there is an evidence of raise in CRP among CVD patients which is to be dealt in detail in the near future to use CRP proteins as a marker in CVD detection.

Keywords: Atherosclerosis, CRP, Coronary Heart Disease, Cerebrovascular Disease, DAB kit, Peripheral Vascular Disease, Hepatocytes, PBMC, SDS PAGE Gel Electrophoresis.

1. Introduction

The leading cause of death in developing countries is related to cardiovascular and cerebrovascular diseases. It refers to the class of diseases that involve the heart or blood vessels (arteries and veins). Cardiovascular diseases include coronary heart disease (heart attacks), cerebrovascular disease, raised blood pressure (hypertension), peripheral artery disease, rheumatic heart disease, congenital heart disease and heart failure death and are projected to remain so. Atherosclerosis of the major arteries is present universally in young adults at autopsy and appears to start early in childhood. The basic mechanism of this

disease can be stated as “a mild chronic inflammatory reaction to injury.”

Inflammation is now widely recognized as a central feature of atherogenesis and it plays a particularly critical role in the destabilization of the fibrous cap, predisposing to the plaque rupture that triggers most episodes of coronary thrombosis. C-reactive protein (CRP) the classic acute-phase reactant is an extremely sensitive systemic marker of inflammation; increased concentrations of CRP have been shown in both clinical and epidemiological studies to be associated with Atherothrombotic events.

C-reactive protein (CRP) was formerly considered solely as a biomarker for inflammation and is now

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viewed as a prominent partaker and independent predictors of cardiovascular disease (CVD). C-reactive protein (CRP) is a plasma protein, an acute phase protein produced by the liver and by adipocytes.

CRP is a marker for metabolic disturbances associated with an increased risk for cardiovascular disease. It has been well established that several of the metabolic changes that characterize insulin resistance and type 2 diabetes, such as a high body mass index, hypertension, hypertriglyceridemia, and low HDL cholesterol are associated with increased CRP levels. The biological explanation for this association remains to be fully elucidated but may involve an increased release of cytokines from adipose tissue. If this alternative is correct, CRP is only a marker of metabolic disturbances increase the risk for development of cardiovascular disease but has in itself no association with the actual cardiovascular disease process (3). CRP is actively contributing to the progression of atherosclerosis. Genetic, metabolic and other factors stimulate the expression of CRP in the liver and possibly also in the arterial wall. CRP will bind to lipoproteins and damaged cells in atherosclerotic plaques induce complement activation thus promoting inflammation and disease progression.

In this work, the serum samples were collected from the hyperinsulinemia patients and also in the normal and is compared based on the estimation of CRP by using the techniques such as Bradford's method of protein estimation, SDS-PAGE and Western blotting. The Insulin levels were estimated using ELISA technique and were compared with the normal samples. The PBMC'S were cultured in RPMI 1640 medium, the protein was isolated and ran on SDS-PAGE. The molecular weight of the protein was compared with the marker protein band.

2. Materials and Methods

2.1 Sample Collection

Blood samples of 20 CVD patients were collected from the Kilpauk Medical College and Hospital, Chennai and also blood samples of 20 healthy volunteer normal subjects were obtained from University centers, Palavakkam, Chennai. These samples were obtained after a minimum 6 hours fast for the measurement of serum CRP levels. After an overnight fast, venous blood samples were collected in CVD subjects and in controls, in the morning between 7.00 - 8.00 am. Blood samples were taken in sterile centrifuge tubes. These tubes were marked with patients name or number for easy identification and date of collection. Then the sample tubes were immediately placed in the icebox until the samples were centrifuged.

During the same visit, all subjects underwent measurements including body weight, height, waist circumferences and blood pressure.

2.2 Serum Separation

Centrifuge tubes containing the blood samples drawn from the CVD patients and normal were centrifuged at 3000 rpm for 10-15 minutes. The serums, thus separated were taken in a sterile eppendorfs. Each serum sample was made into different aliquots and stored at -80°C until analysis. This is done to avoid repeated freezing and thawing of serum samples and also to prevent haemolysis, contamination and heat inactivation which may cause erroneous results.

2.3 BMI Measurements

The classification of overweight and obesity by percentage of body fat, body mass index (BMI), waist circumference and associated disease risk is tabulated in Table 1.

BMI = Weight (in Kg)/Square Height (in meters)

	WEIGHT RANGE	BMI [kg/meter square]
	Underweight	<18.5
	Normal weight	18 - 24
	Overweight	25 - 29.9
i.	Obese	30 - 35
ii.	Obese	35 - 39
iii.	Extremely obese	≥ 40.

The body weight and height of the patients were measured. BMI were calculated by dividing weight (in kilograms) by the square height (in meters).

2.4 Estimation of Biochemical Profiles

The biochemical parameters such as triglyceride, HDL and cholesterol were analyzed using standard methods. Insulin values were measured using INSULIN ELISA KIT [Calbiochem Inc. USA].

2.5 Estimation of serum insulin using ELISA technique

Insulin ELISA was used for the quantitative determination of insulin levels in serum of CVD patients and controls.

In this procedure, immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well, a serum contains the native antigen and exogenously added biotinylated monoclonal anti-insulin antibody, to form a soluble sandwich complex. The enzyme activity in the antibody bound fraction is directly proportional to the native antigen concentration.

2.6 Peripheral Blood Mononuclear Cells

2.6.1 Medium

- RPMI 1640 medium supplemented with 200 mM L-glutamine (Sigma St. Louis, USA).

- b. Ficoll-hypaque medium (Lymphoprep, Nyegaard and Denmark).
- c. 10% heat inactivated fetal bovine serum (Sigma, St. Louis, USA).
- d. Phytohemagglutinin (PHA) was purchased from Bangalore Genei, India. PHA (1mg/ml) was dissolved in of phosphate buffered saline (pH 7.2).
- e. Leucosep tubes for the separation of peripheral mononuclear cells from human whole blood (Greiner Bio-One, Germany).
- f. Strep well insulin ELISA kit (Calbiochem Inc. USA).

2.6.2 Methods

Normal human peripheral blood samples (5ml) were obtained from healthy volunteers, ranging between 25 to 30 years of age. Written informed consent was obtained from all volunteers.

2.7 Isolation of PBMC from peripheral blood

PBMC were isolated according to the instruction manual with slight modifications. 3ml of Ficoll-Hypaque medium (Lymphoprep, Nyegaard and Denmark) was added to the Leucosep and centrifuged for 30 seconds at 4000 rpm at room temperature, in order to locate the medium below the porous barrier than 5ml of anticoagulated blood was poured carefully from the blood sampling tube into the Leucosep tube. Samples were centrifuged for 15 minutes at 4000 rpm at 260°C in a swinging bucket rotor. The enriched cell fraction was then harvested by Pasteur pipette into another centrifuge tube and washed thrice with PBS. Cells were then carefully transformed into 1ml of RPMI 1640 medium and centrifuged at 1500 rpm for 15 Minutes. The supernatant was discarded and the pellet was suspended in RPMI 1640 medium and mixed gently by aspiration, washed twice and resuspended in 0.5 ml of fresh RPMI 1640 medium.

2.8 PBMC culture

The isolated PBMC in 0.5ml of the medium was made up to 5ml with RPMI 1640 medium supplemented with L-glutamine, 100 Iu/ml penicillin, 100µg/ml streptomycin and 1ml of 10% heat inactivated fetal bovine serum (FBS) was added. This mixture is designated as “complete medium” (Boscoco *et al.*, 2003). PBMC was then allowed to grow in unstimulated condition (absence of PHA) at 370°C for 72 hours.

2.9 PBMC culture in stimulating condition

The isolated PBMC in 0.5ml of the medium was made up to 5ml with RPMI 1640 medium supplemented with L-glutamine, antibiotics (100 Iu/ml penicillin, 100µg/ml streptomycin) and ml of 10% heat inactivated fetal bovine serum (FBS) was added. The PBMC was allowed to grow in stimulating condition

(0.2ml of 10µg/ml PHA and insulin 40µg per 5ml) at 370°C for 72 hours.

2.10 Protein isolation

After 72 hours of incubation at 370°C, the culture cells lysed using RIPA buffer and the protein were separated by the ultrasonication method. Protein thus obtained was then estimated using the following protocol (Bradford reagent method).

2.11 Protein estimation by using Bradford's method

This is a rapid and accurate method for estimating the protein concentration when compared with Lowry's method. It subjects a very less interface by common reagents and nonprotein components of biological samples.

The principle of this method relies on the binding of the dye Coomassie brilliant blue G250 to the protein. The quantity of the protein can be estimated by determining the amount of dye in the blue ionic form. This is usually achieved by measuring the absorbance of the solution at 595 nm or 625 nm. The standard protein used is BSA.

$$\text{Sample concentration} = x/v \text{ mg /ml}$$

Where, x - value from graph in µg;

v - Volume of sample in µl.

2.12 Analysis of CRP using SDS page electrophoresis

SDS PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) is the most widely used methods for analyzing protein mixtures. Since the method is based on the separation of proteins, according to the size, mass and charge, it can also be employed for the determination of relative molecular mass of proteins. It is often used after each step of the purification protocol to assess the purity of the sample. Pure protein should give a single band unless the molecule is made up of two unequal subunits.

Sodium dodecyl sulfate is a very strong anionic detergent. It is an amphipathic molecule that consists of non-polar hydrophobic region and a strong anionic group. In the presence of SDS and a reducing agent such as β-mercaptoethanol, oligomeric proteins are dissociated into their constituent polypeptide chains.

The separating gel used to be a 15% polyacrylamide gel. This gives the gel of a certain pore size in which, the proteins in the range of 20-80 kDa were separated without any hindrance. The stacking gel used to be off 5% in concentration.

The serum samples of the 5 CVD patients and 5 healthy samples were used to study the expression of CRP using SDS-PAGE. The size of the protein ranged near 23-25 kDa. The samples were run along with a protein marker CPM-1 ranging from 6.5-70 kDa for comparing the transfer of the proteins along the gel.

2.13 Western Blot Assay

The western blotting or immunoblotting is an analytical technique which is used to detect specific proteins in a given sample. Immunoblotting is used to identify and measure the size of macromolecular antigens that react with a specific antibody. The proteins are first separated by electrophoresis through SDS-polyacrylamide gels and then transferred electrophoretically from the gel to a solid support, such as nitrocellulose, polyvinylidene difluoride [PVDF] and cationic nylon membrane. After the unreacted binding sites of the membrane are blocked to suppress nonspecific adsorption of antibodies, the immobilized proteins are reacted with a specific polyclonal or monoclonal antibody. Antigen-antibody complexes are finally located by radiographic, chromogenic and chemiluminescent reactions.

DAB (Diaminobenzidine tetrahydrochloride) system was tested in detection of horseradish peroxidase.

3. Results and Discussion

The age and sex of 20 CVD patients and 20 healthy volunteer normal subjects from whom the blood samples were collected is represented in Table 1.

The body mass index value of the CVD patients was calculated and is shown in Table 2.

To correlate the biochemical profiles with the diagnosis of CVD the triglycerides, HDL, cholesterol, and insulin level was assayed and are shown in Table 3a & 3b.

(Serum insulin level was assayed using ELISA method).

Table 1. List of the normal patient and CVD patient age and sex details.

No. of the Normal Patient	Age	Sex	No. of the CVD Patient	Age	Sex
1.	34	F	1.	41	M
2.	57	M	2.	56	M
3.	31	M	3.	30	M
4.	58	F	4.	45	M
5.	42	M	5.	75	M
6.	95	M	6.	45	M
7.	49	F	7.	45	M
8.	41	M	8.	55	M
9.	68	F	9.	55	F
10.	65	F	10.	62	M
11.	36	M	11.	46	M
12.	38	F	12.	46	M
13.	65	F	13.	56	F
14.	58	M	14.	58	M
15.	45	F	15.	54	F
16.	52	F	16.	55	M
17.	56	M	17.	35	M
18.	47	F	18.	64	M
19.	48	F	19.	70	F
20.	31	M	20.	60	M

Table 2. List of BMI in both CVD and normal sample.

No. of the Normal Patient	BMI	No. of the CVD Patient	BMI
1	32.4	1.	25.41
2	28	2	23.2
3	32.8	3.	22.61
4	26.8	4.	38.05
5	29.4	5.	29.61
6	25.4	6.	26
7	28.7	7.	24.1
8	24.1	8.	19.81
9	34.1	9.	23.81
10	30.1	10.	28.05
11	21.3	11.	24
12	30.1	12.	22.77
13	37.7	13.	23
14	24.6	14.	23.71
15	31.6	15.	20.54
16	30.2	16.	21.3
17	30.1	17.	18
18	41.6	18.	26.81
19	27.4	19.	32.44
20	26.9	20.	24

Table 3a. Lipid profile and insulin values of CVD sample.

No. of the Normal Sample	Lipid Profile			Insulin (μ /ml)
	TGL (mg/dL)	CHO (mg/dL)	HDL (mg/dL)	
1	118	236	48	17.7
2	98	216	43	15.9
3	80	221	44	30
4	126	232	46	15.2
5	69	172	34	14.5
6	86	260	43	14.6
7	108	207	43	27.8
8	96	230	45	26.7
9	79	196	30	23.7
10	80	236	47	17.8
11	108	203	40	16.2
12	126	218	44	13.6
13	108	246	48	25.3
14	86	182	36	24
15	107	238	48	27.2
16	102	228	46	16.2
17	108	230	46	29
18	78	222	44	30.2
19	140	230	46	10.8
20	199	254	51	22.2

Table 3b. List of lipid profile and insulin values in normal sample.

No. of the CVD Patient	Lipid Profile			Insulin (μ /ml)
	TGL (mg/dL)	CHO (mg/dL)	HDL (mg/dL)	
1.	286	315	39	39.1
2	121	157	40	43.6
3.	143	190	35	28.8
4.	179	200	44	37.5
5.	390	280	39	29.2
6.	197	169	44	32.7
7.	121	157	40	45.7
8.	198	236	37	34
9.	164	197	39	54
10.	215	269	30	34
11.	199	270	38	37
12.	159	107	40	34
13.	163	188	38	34
14.	179	128	35	44
15.	175	190	40	35
16.	287	220	44	32
17.	215	187	52	33
18.	175	225	44	31
19.	168	120	35	29.5
20.	198	218	40	37.5

Table 4. Bradford's method of protein estimation (Standard BSA estimation - values measured at OD).

Standard BSA Concentration [μ g/ml]	OD at 595 nm	Control Serum Sample	OD at 595 nm	Patient Serum Sample	OD at 595 nm
10	0.269	1	0.623	1	0.996
20	0.547	2	0.544	2	0.805
40	0.840	3	0.630	3	0.668
60	0.650	4	0.644	4	0.722
80	0.709	5	0.705	5	0.633

The peripheral blood mononuclear cells were isolated from the samples obtained and was cultured under simulated condition after cultivation the cells were lysed and the protein was extracted and estimated by Bradford's method and also a serum sample of CVD patients and normal samples were estimated. The values are shown in Table 4.

The relationship of CRP level with CVD was as deduced by production using SDS PAGE Electrophoresis.

C-reactive protein is a nonspecific marker of systemic inflammation that predicts incident Type II diabetes. The metabolic syndrome is based on insulin resistance, hypertension, impaired glucose tolerance, hyperinsulinemia, increased level of triglycerides, decreased level of HDL and cholesterol.

The metabolic syndrome is associated with a greatly increased risk of cardiovascular disease. Circulating CRP only reflects the extent of nonspecific stimuli such as smoking, vascular injury, necrosis, infectious agents and atherosclerosis. Elevated levels of CRP are known to predict the development of Type II diabetes and cardiovascular disease. Elevated CRP levels have been cross-sectionally associated with proxy indicators of elevated body fatness [Body weight and BMI] and with cardiovascular disease risk factors and insulin resistance.

Venous blood samples were drawn from 20 CVD patients and also from normal. The samples were stored at -80°C until assay. Total cholesterol and triglycerides were determined enzymatically. HDL was measured by phosphotungstate method and LDL also measured by the enzymatic method. Insulin levels were estimated by using INSULIN ELISA method. The insulin values were read at ELISA reader 454 nm. The normal insulin level in human being 5-25mg/dL and compared to high level of insulin in diseased patients due to a diabetic's disorder occurs. In case of hyperinsulinemia patient high level of insulin occurs.

Measurements of age, sex, BMI, blood pressure, smoking, coronary heart disease and diabetes status were measured at mean ages 50.8 and 52.65

The serum sample of CVD patients and normal patient sample was estimated through protein estimation and analysis of CRP using SDS PAGE gel electrophoresis. The colored protein marker 6.5-70 kDa was used to identify the CRP protein (25 kDa) and confirm the expression of CRP in both CVD and normal. In CVD patients, CRP expressed in high level & in normal cases the level of CRP expression is low.

Other methods, CRP protein assayed by western blot assay (Immunoblotting) and based on the color development system (DAB) using transfer of protein in SDS gel to nitrocellulose membrane. The membrane

developed that color band after the addition of primary and secondary antibody. The color band membrane shows that figure.

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